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PRINCIPAL INVESTIGATOR: Konstantina Alexandropoulos, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

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Konstantina Alexandropoulos, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

E-MAIL:
ka141@columbia.edu

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13. ABSTRACT *(Maximum 200 Words)*

Strong evidence exists that the c-Src non-receptor tyrosine kinase plays a role in the pathology of human breast tumors. The purpose of this study is to examine the role of c-Src in mammary tumorigenesis and elucidate the mechanisms that lead to tumor formation in an animal model for breast cancer. In our previous experiments we used Src substrates that we cloned to activate c-Src and study its signaling mechanisms. Using one of these substrates, Sin, we characterized a signaling cascade that is activated as a result of Sin binding to Src and Src-mediated Sin phosphorylation. We found that Src-phosphorylated Sin recruits a signaling complex that leads to the activation of the small GTP-binding protein Rap1. Rap1 then activates the ERK kinase that in turn mediates Src-dependent transcriptional activation. In addition, when we compared Sin-activated wild-type Src and an oncogenic Src protein we found that their signaling mechanisms differed in that, wild type Src signaling is mediated by the Rap1 cascade whereas oncogenic Src signaling is mediated by Rap1, as well as another G-protein, Ras. Our results for the first time implicate the Rap1 pathway in wild type and oncogenic Src signaling and reveal mechanistic differences in the signaling mechanisms of these proteins. In our future experiments we will address the role of Rap1 activation in cellular transformation and mammary tumorigenesis in cell lines and transgenic mice coexpressing Src and Sin. These experiments will provide insight into the mechanisms of Src-mediated tumorigenesis and may lead to the identification of proteins that will be used as targets for drug development.

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INTRODUCTION

We are studying the role of the non-receptor tyrosine kinase c-Src in mammary tumorigenesis in a murine animal model. Our purpose is to use c-Src substrates that we have isolated to gain insight into the mechanisms that regulate the activity of c-Src and define the molecular interactions that mediate Src-dependent intracellular signaling and transformation. The molecules we developed bind to a conserved regulatory region of c-Src activate Src's enzymatic activity and subsequently act as substrates and effector molecules for c-Src signaling. In our experiments we will focus on one novel protein that we isolated, Sin, to identify factors which play a role in Src-mediated tumorigenesis in the mammary epithelium. We will focus on Sin because this protein has the unique ability among the other c-Src ligands to activate the transforming potential of c-Src. Thus, in our studies we will a) express c-Src and Sin in the mammary epithelium to assess the effect of these proteins on mammary adenocarcinomas and b) identify Sin sequences that are important for Sin function and use these sequences as a means to isolate proteins that mediate Sin-dependent c-Src tumorigenesis. Given that c-Src has been implicated in the development of human breast tumors, these studies are important because they will elucidate the molecular mechanisms that drive mammary tumorigenesis and lead to the development of strategies to interfere with aberrant Src activity and Src-induced carcinogenesis.

BODY

c-Src is a non-receptor tyrosine kinase that is very important for cellular function (2). The Src substrate Sin that we have characterized is a multi-adapter molecule that mediates the formation of multi-protein complexes in a phosphotyrosine-dependent manner (1). Because phosphorylated Sin has the ability to bind to different proteins simultaneously, it also has the potential for activating multiple intracellular pathways with pleiotropic effects on cellular behavior. In our preliminary results we found that a truncated version of Sin, SinΔC, activates two major signaling pathways and the transforming potential of c-Src.

Given the effects of SinΔC on Src activation, in aim 1 of the application we proposed to generate transgenic mice coexpressing Src and SinΔC in their mammary epithelium and examine whether expression of these proteins would result in tumors.

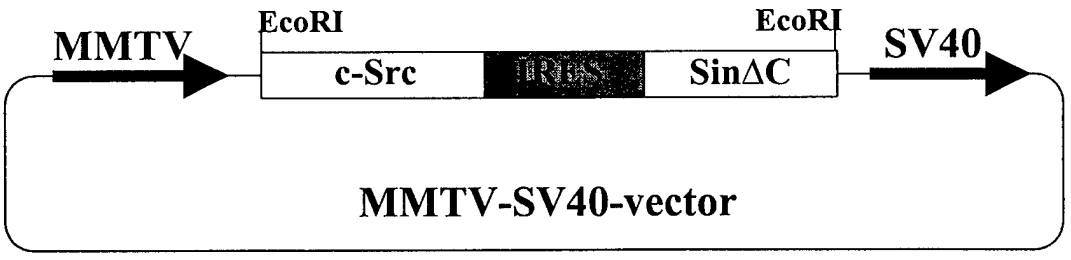

Fig. 1. *Transgenic construct coexpressing c-Src and SinΔC.* This construct is generated by inserting an EcoRI DNA linear fragment containing Src- and SinΔC-expressing cDNAs as well as an internal ribosome entry site (IRES) that allows coexpression of Src and SinΔC, into the EcoRI linearized MMTV vector. MMTV is the long terminal repeat of the mouse mammary tumor virus that allows expression of cloned cDNAs and SV40 is the polyA/splice of SV40.

We are now in the process of generating this transgenic construct. A linearized fragment of the MMTV vector containing the sequences MMTV-c-Src-IRES-SinΔC-SV40 will be microinjected into fertilized eggs from hyperovulated donor females. Transgenic progeny will be identified by analyzing mouse tail DNA by PCR and Southern blots using Src- and SinΔC-specific primers. These animals will then be bred and the F1 progeny will be observed for tumor formation as described in the original application.

In aim 2 of the original application we proposed to analyze the sequences of Sin to identify elements the are important for the signaling properties of the protein and use these sequences as probes to identify proteins that mediate Sin-dependent Src signaling. In a manuscript included in the appendix we have now characterized the signaling mechanisms of Sin and have identified proteins that are involved in mediating Src signaling using a cell culture system (3). In this study, through the use of mutagenesis analysis and dominant negative inhibitors, we identified three Sin tyrosine motifs (Y motifs) that are important for Src signaling. These Y-motifs contain the conserved amino acid sequence YDVP, become phosphorylated by Src and are important for Src-dependent transcriptional activation. We also found that these motifs are required for the recruitment of a signaling complex that consists of the adapter protein Crk and C3G, a nucleotide exchange factor that promotes guanyl-triphosphate (GTP) binding on the GTP-binding protein Rap1. Binding of GTP to Rap1 activates this protein. Rap1 then activates another kinase ERK1 that in turn is required for transcriptional activation. Activation of both Rap1 and ERK1 is required for Sin-mediated Src signaling. Our results for the first time implicate this pathway in c-Src signaling and provide insight into the signaling mechanisms of wild type Src.

The effect of activation of the Rap1 pathway on cellular transformation and mammary tumorigenesis will be explored first in cell lines and then in c-Src/SinΔC transgenic mice. Although our data show that the Y-motifs of Sin, described above, are required for Src signaling in the form of transcriptional activation, at the present time it is not clear whether these motifs and subsequent Rap1 activation are required for Src dependent, Sin-mediated transformation. We are currently testing this possibility in cell cultures using a SinΔC mutant with point mutations on the three YDVP-containing motifs that substitute the tyrosine residues with phenylalanine (SinΔC-TM). If these motifs are important for Sin-mediated Src transformation we should observe no transformation of NIH3T3 cells expressing c-Src and SinΔC. The effect of this mutant will then be expressed in the mammary epithelium of transgenic mice expressing c-Src and SinΔC-TM. If these Y-motifs of Sin are required for Sin-mediated Src transformation we should observe no tumor formation in these mice. If it turns out the Sin-mediated activation of the Rap1 pathway is required for tumorigenesis, Rap1 will then serve as a good target for therapeutic approaches and drug design. If cellular transformation and tumors are still observed, we will then perform additional mutagenesis on Sin. These sequences will then be used as probes to identify proteins that bind to them as described in the original proposal to identify sequences that abolish transformation.

KEY RESEARCH ACCOMPLISHMENTS

1. We have shown that Sin interaction with Src leads to the activation of Src signaling as assayed by transcriptional activation.

2. We have for the first time described a novel pathway that mediates wild type c-Src signaling.
3. We have characterized the components of this pathway. These include Src/Sin, the signaling complex Crk/C3G, the G-protein Rap1 and the kinase ERK1.
4. Our experiments have revealed mechanistic differences in the signaling mechanisms of wild type versus transforming Src alleles.

REPORTABLE OUTCOME

-We have published a manuscript under the title "c-Src Signaling induced by the adapters Sin and Cas in mediated by Rap1 GTPase". 2000. Mol. Cell. Biol. Vol. 20: p. 7363-7377.

An oral presentation and an abstract were presented in an annual meeting: "Tyrosine Phosphorylation and Cell Signaling: The Third Decade". August 9-13, 2000, The Salk Institute, San Diego, CA.

An oral presentation was given at the FASEB annual meeting: "FASEB 2000, Signal Transduction in the Immune System". Saxton River, VT.

-No degrees obtained

-We have developed cell lines coexpressing Src and SinΔC using NIH3T3 cells.

-No informatics

-We have applied for funding from the National Institute of Health (NIH) on work supported by this award which are pending. These are:

RO1 AI49387 $1,125,000
Title: Examine the Role of Fyn and Rap1 in T cell activation and T cell-mediated Immune Responses.

RO1 AI48558 $1, 125,000
Title: Assessment of the role of the Rap1 GTPase in Thymocyte development.

-No changes in employment status.

CONCLUSIONS

Oncogenic Src proteins have been extensively studied to gain insight into the signaling mechanisms of Src. To better understand signaling through wild-type Src, we used an approach that involves activation of Src signaling through the binding of physiologic ligands to the Src SH3 domain. To this end we used full length and truncated versions of the multiadapter Sin to activate c-Src, and we examined the intracellular pathwyas that mediate Src signaling under these conditions. We found that Sin-induced Src signaling,

as assayed by transcriptional activation, is exclusively mediated through a pathway that involves the adapter Crk and the GTP-binding protein Rap1. Activation of the Rap1 pathway by Sin was mediated by three conserved sequences within Sin that promote association of Src-phosphorylated Sin with the Crk/C3G/Rap1 signaling complex. These data are in contrast to previous observation showing that another GTP-binding protein, Ras, mediates signaling downstream of transforming Src alleles. In our system we found that signaling through an oncogenic Src allele is indeed mediated by Ras. In addition, we found that Rap1 also mediates oncogenic Src signaling.

These results are important because for the first time implicate the Rap1 pathway downstream of activated wild-type Src and provide insight into the signaling mechanisms of this protein. Our results are also important because for the first time they reveal mechanistic differences in the signaling properties of wild-type and transforming Src alleles. Insight provided by these experiments into the molecular mechanisms of Src signaling will be of great value in addressing the signaling events that lead to Src dependent transformation and tumorigenesis in the mammary epithelium.

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APPENDIX

MOLECULAR AND CELLULAR BIOLOGY, Oct. 2000, p. 7363–7377 Vol. 20, No. 19
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c-Src Signaling Induced by the Adapters Sin and Cas Is Mediated by Rap1 GTPase†

LUZHOU XING,[1] CHANG GE,[1] ROSS ZELTSER,[1] GREGORY MASKEVITCH,[1] BRUCE J. MAYER,[2] AND KONSTANTINA ALEXANDROPOULOS[1]*

Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York 10032,[1] and The Children's Hospital, Boston, Massachusetts 02115[2]

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Oncogenic Src proteins have been extensively studied to gain insight into the signaling mechanisms of Src. To better understand signaling through wild-type Src, we used an approach that involves activation of Src signaling through the binding of physiologic ligands to the Src SH3 domain. To this end, we used full-length and truncated versions of the multiadapter molecules Cas and Sin to activate c-Src, and we examined the intracellular pathways that mediate Src signaling under these conditions. We show that although all proteins bind to and are phosphorylated by c-Src, quantitative differences exist in the ability of the different ligands to activate c-Src signaling. In addition, we show that Sin- and Cas-induced Src signaling, as assayed by transcriptional activation, is exclusively mediated through a pathway that involves the adapter Crk and the GTP-binding protein Rap1. These data are in contrast to previous observations showing Ras to mediate signaling downstream of transforming Src alleles. In our system, we found that signaling through the oncogenic SrcY527 mutant is indeed mediated by Ras. In addition, we found that Rap1 also mediates oncogenic Src signaling. Our results show for the first time that Rap1 mediates c-Src kinase signaling and reveal mechanistic differences in the signaling properties of wild-type and transforming Src proteins.

The nonreceptor protein tyrosine kinase Src is critical for normal cellular processes such as proliferation and differentiation, and certain mutations in Src cause uncontrolled cell proliferation and transformation (11). Under normal conditions, the enzymatic activity of Src is tightly regulated. Biochemical (13, 20, 45, 64) and structural (75, 92) analyses have shown that the kinase activity of the c-Src protein is intramolecularly regulated by conserved modular domains, the Src homology regions 2 and 3 (SH2 and SH3) (18). Consistent with their regulatory role, mutations within these domains render the kinase active and oncogenic (11). In addition, upon Src activation, these domains mediate protein-protein interactions and are thought to determine substrate selectivity and signaling specificity (18, 28).

Traditionally, studies aimed at elucidating the signaling properties of c-Src have used constitutively active and transforming Src alleles as models. Activated Src alleles exhibit deregulated kinase activity and are known to induce multiple signaling responses due to promiscuous substrate phosphorylation. Thus, it has been difficult to determine which of the many responses is responsible for the signaling properties of Src. In addition, despite the identification of a plethora of putative Src substrates in v-Src-transformed cells, the importance of these substrates in the physiologic and/or tumorigenic effects of c-Src has been difficult to ascertain.

To gain insight into the signaling mechanisms of wild-type c-Src and given that the c-Src SH3 domain has been shown to participate in the intramolecular negative inhibition of the c-Src kinase activity (55, 79), we used physiological ligands for the conserved SH3 domain of c-Src to activate the enzyme. At the same time, we used these ligands as links to downstream events to study the signaling mechanisms and specificity of c-Src. The molecules used for our studies consist of a protein that we previously identified, Sin, and the homologous protein $p130^{Cas}$ (1, 72). Cas was first identified as a highly phosphorylated protein in v-Src- and v-Crk-transformed cells (72); Sin was independently cloned as the Fyn embryonic substrate Efs (40). These molecules specifically bind to Src family SH3 domains with high affinity through proline-rich motifs (2, 57, 72). Sin and Cas comprise a multiadapter protein family that also includes HEF1/CasL independently cloned as a human enhancer of filamentation in yeast and as a focal adhesion kinase (FAK)-binding protein expressed in lymphocytes (48). All of these proteins exhibit conserved secondary structures, which in turn consist of many conserved modules that mediate protein-protein interactions. Thus, Cas proteins have conserved N-terminal SH3 domains, central regions comprised of repeated tyrosine-containing residues, Src SH3-binding proline-rich motifs (except HEF1/CasL), and conserved C termini that have been implicated in homo- or heterodimerization between family members (61). The presence of these conserved domains and their ability to promote protein-protein interactions suggest that members of the Cas family mediate the formation of multiprotein complexes in a phosphotyrosine-dependent manner. These protein-protein interactions are thought to subsequently activate intracellular signaling pathways with pleiotropic effects on cellular behavior (52, 61).

The most extensively studied member of this family, $p130^{Cas}$, becomes highly phosphorylated on multiple tyrosine residues in response to a variety of stimuli. For example, mitogens such as epidermal growth factor, platelet-derived growth factor, and lysophosphatidic acid have been shown to induce tyrosine phosphorylation of Cas (15, 59). In addition, integrin engagement or stimulation of serpentine receptors such as the bombesin and the endothelin receptors stimulate Cas phosphorylation (15, 47, 87, 88). Cas phosphorylation in turn has been implicated in multiple cellular processes such as integrin re-

* Corresponding author. Mailing address: Department of Pharmacology, College of Physicians and Surgeons of Columbia University, PH 7W Rm. 318, 630 West 168th St., New York, NY 10027. Phone: (212) 305-2705. Fax: (212) 305-8780. E-mail: ka141@columbia.edu.

† This report is dedicated to the memory of Eugenia Spanopoulou, Andrew Hotchev, and Platon Spanopoulos-Hotchev. Δεν σας ξεχνω.

ceptor signaling (36, 50, 58, 88), cell migration and survival (14, 16, 17, 44), regulation of the cell cycle (60, 93), and apoptosis (7). Furthermore, Cas has been implicated in cellular transformation, as demonstrated by its presence as a tyrosine-phosphorylated protein in v-Src- and v-Crk-transformed cells (72), by the fact that $p130^{Cas-/-}$ cells cannot be transformed by Src (37), and by antisense RNA experiments showing that Cas-specific antisense RNA partially reverts v-Src transformation (6).

Multiadapter molecules, such as the Cas protein, depending on the type and number of conserved motifs they contain, can form interactions with unique sets of cytoplasmic intermediates and thus determine signaling specificity. These conserved motifs have tyrosine-containing sequences (Y motifs) which upon phosphorylation by tyrosine kinases recruit cytoplasmic substrates through phosphotyrosine-SH2 domain interactions. The type and number of Y motifs present on the different members of the Cas family differ, suggesting that these proteins may have similar but not identical functions. Consistent with the model that Y motifs can determine signaling specificity, the multiadapters and insulin receptor substrates IRS1 and -2 (56, 94) have been shown to mediate distinct functions of the insulin receptor (5, 80, 89). The different signaling properties of each molecule have been attributed to unique Y motifs that bind to distinct SH2 domain-containing cytoplasmic intermediates.

We have previously shown that coexpression of Src and Sin activates Src signaling, as measured by serum response element (SRE)-mediated transcriptional activation (1). In this study we compared the abilities of the Src SH3-binding proteins Cas and Sin to bind to and stimulate the enzymatic activity of c-Src. We then analyzed the molecular events that mediate Sin-Cas-induced Src signaling, using the phosphorylated forms of these proteins as links to downstream signaling events. In addition, we compared the signaling mechanisms of ligand-activated c-Src to signaling mediated by a constitutively active and oncogenic Src mutant. In these experiments, we used full-length and truncated forms of Sin and Cas proteins, and we found quantitative differences in the ability of the different Sin and Cas proteins to activate c-Src signaling. In addition, we found that ligand-activated and constitutively active Src proteins utilize different signaling mechanisms to induce SRE-dependent transcription.

MATERIALS AND METHODS

Cells and antibodies. Human embryonic kidney carcinoma 293 cells were grown as previously described (62). Cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The Sin monoclonal antibody, raised against a fragment of Sin encompassing amino acids 142 to 258, was generated by Transduction Laboratories. Cas polyclonal antibody CasN-17 was purchased from Santa Cruz Biotechnology. The Src mouse monoclonal antibody 327 was provided by Joan Brugge (Harvard Medical School); the antiphosphotyrosine monoclonal antibody pY20 was purchased from Transduction Laboratories. Antiphosphotyrosine SrcY416 was kindly provided by A. Laudano (University of New Hampshire). Other reagents used were anti-phospho-ERK monoclonal antibody (E-4), anti-ERK, anti-C3G (Santa Cruz), anti-Ras, anti-Rap1, anti-Crk (Transduction Laboratories), and anti-hemagglutinin epitope (HA) (BAbCo, Richmond, Calif.).

DNA constructs. DNA manipulations were performed by standard protocols. Full-length Sin and truncated SinΔC (amino acids 1 to 335) were cloned into the *Spe*I-*Not*I sites of the pEBB expression vector. pEBB was derived from the pEF-BOS expression vector driven by the human elongation factor 1-α promoter (53). In SinΔC, a deletion of amino acids 340 to 560 removes one of the proline-rich Src SH3-binding sites, the last three Y motifs in the substrate region, and the C-terminal homologous region. PCR-amplified Cas proteins were cloned into the *Bam*HI-*Not*I sites of pEBB. Full-length Cas expresses a short form of the protein that is alternatively spliced, missing amino acids 5 to 99 (72). CasΔUR contains an internal deletion of a *Bsu*36I fragment encompassing amino acids 496 to 713 that removes the unique region of Cas. CasΔURΔC contains, in addition to the *Bsu*36I fragment deletion, a C-terminal deletion that encompasses amino acids 748 to 968. This construct was generated by PCR amplification using CasΔUR as a template and synthetic oligonucleotides containing 5′ *Bam*HI and 3′ *Not*I restriction sites. CasΔURΔCP* was generated as the CasΔURΔC construct except that the 3′ oligonucleotide that was used for PCR amplification contained two base pair substitutions that produce two point mutations (Ser-Ala and Pro-Leu) in the sequence within the core of the PXXP motif of Cas. These substitutions change the Src SH3-binding site of Cas for that of Sin (PSPP to PALP). All sequences were confirmed by automated sequence analysis. The different SinΔC YDVP mutants were generated by PCR by substituting tyrosines 148, 188, and 253 with phenylalanine residues, using mutated oligonucleotides. The amplified fragments were cloned into the *Spe*I-*Not*I sites of pEBB, and mutations were confirmed by automated sequencing. Plasmids expressing the RasN17 and CrkW170K mutants were previously described (30, 81). The RapN17 mutant was provided by Philip Stork (Oregon Health Sciences University) (86). The SRE-luciferase reporter construct was generated as previously described (1); the AP-1 luciferase reporter was provided by C. A. Hauser (La Jolla Cancer Research Foundation). Plasmids that expressed wild-type Src protein and SrcY527F have been described elsewhere (45), as has the v-Raf construct (68).

Transfections. 293 cells were transfected as previously described (1, 62). Two micrograms of pMHHB(c-Src) or pc-SrcY527F expression vector or pEVX empty vector was used in all transfections. The transfection mixtures also included 1 μg of vector (pEBB) used to express the different Sin or Cas proteins or 1 μg of Sin- and Cas-expressing pEBB and different concentrations of the inhibitors as shown in the figures. SRE and AP-1 luciferase reporters and the MFG-*lacZ* plasmid expressing β-galactosidase (1 μg of each) were also included in the transfection mixtures. When necessary, the total amount of DNA in each transfection mixture was kept constant by the addition of empty vector in the DNA-calcium phosphate coprecipitate. Luciferase activity was determined using a Promega kit according to the manufacturer's protocol; β-galactosidase activity to normalize for transfection efficiency between the different samples was determined according to standard protocols (73).

Immunoprecipitations. Immunoprecipitations were performed as previously described (1). Briefly, cells were lysed in 1 ml of ice-cold NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml) and incubated on ice for 10 min. The cell debris and nuclei were removed by centrifugation in an Eppendorf centrifuge for 10 min at 4°C. The cell lysates were then incubated with the specified antibodies at concentrations suggested by the manufacturers for 2 h at 4°C. The immune complexes were collected after the addition of 20 μl of protein G-plus–protein A–agarose (Oncogene Science) and incubation at 4°C for 30 min. The pellets of agarose beads were washed three times with 1 ml of lysis buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Western blot analysis. Total cell extracts or immunoprecipitates normalized for protein content were boiled in Laemmli sample buffer, separated by SDS-PAGE on a 10% gel, and transferred to nitrocellulose membranes. Filters were blotted with the appropriate monoclonal antisera according to manufacturer's protocol in TBST-milk at 4°C overnight (16 h). Rabbit polyclonal antibodies were used at 1:500 dilution. Monoclonal antibodies were each used at 1 μg/ml of TBST-milk. The filters were washed in TBST and consequently incubated with anti-mouse or anti-rabbit immunoglobulin G-conjugated horseradish peroxidase at a 1:4,000 dilution in TBST at room temperature for 1 h. Filters were then washed and developed by enhanced chemiluminescence (ECL) (Amersham) as described by the manufacturer.

Ras and Rap1 binding assays. Transfected 293 cells were lysed with cold lysis buffer (50 mM Tris [pH 7.5], 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.5 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride), and aliquots of the cell extracts containing 50 μg of total protein were mixed with 40 μl crude bacterial extracts expressing glutathione *S*-transferase (GST)–RalGDS–RBD (Rap1-binding domain) or Raf1-RBD (Ras-binding domain) that had been precoupled to glutathione beads in the presence of 125 mM NaCl. The aliquots were incubated 1 h at 4°C with rotation. The protein complexes were washed, separated by SDS-PAGE on a 15% polyacrylamide gel, transferred on nitrocellulose membranes, and blotted with Ras- and Rap1-specific antibodies.

RESULTS

Differential activation of Src signaling by Sin and Cas. To gain insight into the specificity of c-Src signaling, we compared the abilities of wild-type and mutated forms of Sin and Cas proteins to bind to and activate Src. Although the Sin and Cas proteins do not share extensive primary sequence homology, they are closely related in their overall secondary structure (Fig. 1). For example, they both contain proline-rich consensus motifs that exhibit high binding affinity to the Src SH3 domain

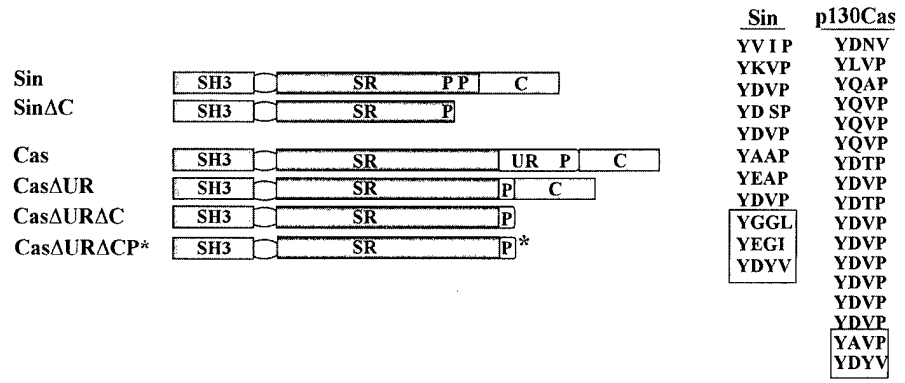


FIG. 1. Schematic representation of full-length and truncated Sin and Cas proteins and comparison of their Y motifs. The constructs were generated as described in Materials and Methods. SR, substrate-binding regions of Sin and Cas that contain the motifs shown on the right; P, proline-rich sequences that bind to the Src SH3, RPLPALP, and RPLPPPP for Sin and RPLPSPP for Cas. Y motifs that are deleted in truncated Sin and Cas proteins are boxed. CasΔURΔCP* is the same as CasΔURΔC except that the PSPP motif has been changed to PALP.

(1, 72) and are important for Src binding. The proline-rich motif of Cas has been shown to mediate association of this protein with Src and is important for Src-dependent phosphorylation of Cas (57). A point mutation within the proline-rich motif of a truncated fragment of Sin or deletion of the Sin proline-rich consensus leads to inhibition of Src signaling (reference 1 and unpublished observations). The N-terminal SH3 domains and the C termini of Cas and Sin are conserved (90 and 57%, respectively), and their central regions contain multiple tyrosine motifs that mediate substrate binding through phosphotyrosine-SH2 domain interactions. In addition, Cas contains a unique region of unknown function between its substrate-binding region and its C terminus. Given the homology of these proteins, we compared the abilities of Sin and Cas to bind to and activate c-Src, as well as their abilities to become phosphorylated and promote Src-dependent intracellular signaling.

To this end, we generated full-length and truncated versions of the two proteins as shown in Fig. 1. The conserved C termini of both Sin and Cas were deleted, given that they negatively regulate the signaling properties of the proteins (see below), as well as the unique region of Cas to more closely approximate the overall structure of Sin. Furthermore, the proline-rich, Src SH3-binding site of Cas was exchanged for that of Sin, to test whether the affinities of these sites for the Src SH3 were functionally equivalent (Fig. 1). Finally, HA-tagged forms of truncated Cas and Sin proteins were generated and compared to unmodified full-length and truncated Cas and Sin proteins. Immunoprecipitation assays revealed that the Sin and Cas proteins associated with c-Src when coexpressed in 293 cells and were phosphorylated on tyrosine residues (Fig. 2A, top panel, lanes 2 to 9). The phosphorylation of the Sin and Cas proteins depended on c-Src coexpression, since only minor or undetectable phosphorylation of these proteins was observed in the absence of Src (pEVX vector) and in the presence of a Src kinase mutant (c-SrcK295R) (Fig. 2A, middle and bottom panels, respectively). These results suggest that these Sin and Cas proteins associate with Src and become phosphorylated in vivo in a Src-dependent manner.

To address whether association of Sin or Cas with Src directly stimulated the enzymatic activity of Src, we generated lysates from 293 cells coexpressing Src and Sin or Src and Cas proteins. Cell extracts were immunoprecipitated with anti-Src-specific antibody and blotted with a polyclonal antiserum that specifically recognized the phosphorylated form of tyrosine 416 of c-Src but not the nonphosphorylated form or other phosphorylated tyrosines (1, 54). Tyrosine 416 is located within the activation loop of the Src kinase domain (92), and its phosphorylation correlates with increased Src activity (45). We found that all of the Cas and Sin proteins that we used were able to induce Y416 phosphorylation to various degrees (Fig. 2B, upper panel, lanes 2 to 9) compared to untransfected control cells or cells transfected with the vector backbone used to express Sin and Cas (pEBB; lane 1). Full-length Cas and a Cas mutant missing only the unique region of the protein (CasΔUR) were less able to induce Y416 phosphorylation when coexpressed with c-Src (Fig. 2B, upper panel, lanes 4 and 5), suggesting that the different proteins, although phosphorylated, differentially induce Src activation. The total amounts of Src were similar in the different samples (Fig. 2B, lower panel), showing that the effects on Y416 phosphorylation were specific and not due to differences in c-Src levels. These data suggest that both Sin and Cas can stimulate the enzymatic activity of Src but that some proteins may be more potent than others in promoting this activation.

Cas and Sin protein expression was confirmed using Sin-, Cas-, and HA epitope-specific antisera (Fig. 2C, upper panel). However, since we used different antisera which may exhibit different affinities for their cognate antigens, we could not directly compare the relative expression levels of the different proteins. To achieve this goal, we used a polyclonal antiserum, CasN-17, which recognizes amino acid residues 105 to 118 of the SH3 domain of Cas and cross-reacts with the Sin SH3 domain. This cross-reactivity was not unexpected because the sequence of the Cas peptide used to generate this antiserum is nearly identical to the corresponding sequence of Sin. Using this antibody, we determined that the different Sin and Cas proteins were expressed in similar amounts (Fig. 2C, lower panel).

We next examined whether Sin and Cas could elicit a signaling response as a result of their association with c-Src as measured by transcriptional activation. We used reporter constructs that drive the expression of luciferase from the SRE of the *egr-1* gene, a c-*fos*-like early-response gene (1, 84), or the AP-1-binding site of c-*jun* (33). The activation of transcription from the SRE promoter is mediated through the Raf/MEK1,2/ERK and serum response factor (SRF) pathways, while AP-1-dependent transcription is mediated by the MEKK/c-Jun N-terminal kinase (JNK) kinase/JNK pathway, as well as in response to increased Fos expression (12, 51). We found that

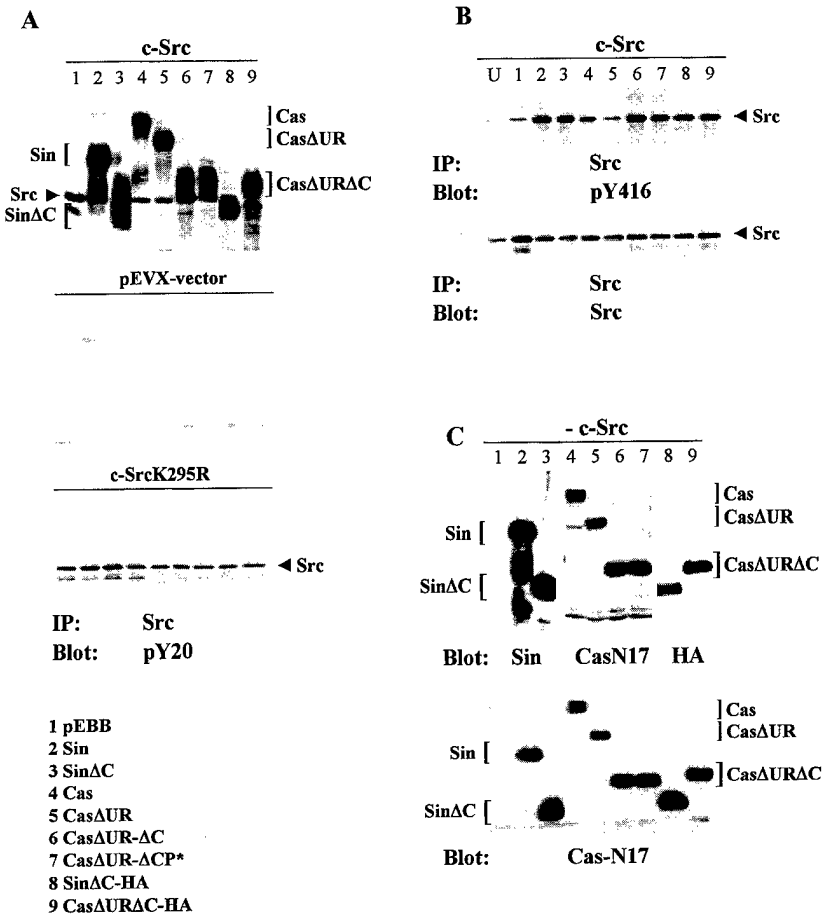


FIG. 2. Sin and Cas associate with and are phosphorylated by c-Src. (A) 293 cells were cotransfected with a full-length or truncated Sin or Cas expression vector (1 μg of each) and a c-Src expression plasmid, the pEVX empty vector used to express Src, or the c-SrcK295R kinase mutant (2 μg of each). Cell lysates were subjected to immunoprecipitation (IP) using anti-Src antiserum, and Western blots of the immunoprecipitates were blotted with pY20, an antibody against phosphotyrosine. In lane 1, cells were transfected with pEBB vector backbone used to express Sin and Cas proteins; lanes 2 to 9 contain the different Sin and Cas proteins as shown at the bottom. (B) 293 cells were transfected with Src and Sin or Cas expression constructs as in panel A. Untransfected controls (U) were included to compare the levels of endogenous versus overexpressed Src. Cell lysates were immunoprecipitated with Src antibody; immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with antibody pY416, which recognizes the active form of Src (top panel). The blots were stripped and reprobed with anti-Src antibody to determine the relative levels of Src in the different lanes (lower panel). (C) The top panel represents total lysates from cells transfected with 1 μg each of the Sin and Cas proteins on a Western blot probed with Sin-, Cas-, or HA-specific antibodies. The bottom panel contains the same extracts blotted with the CasN-17 antibody. Protein bands on the different blots were visualized using horseradish peroxidase-conjugated secondary antibody and ECL reagents. All blots were exposed on film for the same amount of time (10 s).

SRE- and AP-1-dependent transcription was activated 7- and 12-fold, respectively, when c-Src and full-length Sin were coexpressed (Fig. 3A and B, top, lanes 2), whereas full-length Cas had no effect (Fig. 3A and B, lanes 4). On the other hand, SinΔC (Fig. 1A) greatly stimulated Src-dependent activation of both promoters (about 33- and 34-fold [Fig. 3A and B, lanes 3]). Other Sin constructs that contained all the Y motifs as well as the second Src SH3-binding site, but lacked the conserved C terminus, behaved similarly to SinΔC (data not shown), consistent with a negative regulatory role for the C terminus of Sin.

In parallel experiments, we also tested Cas deletion mutants (Fig. 1A) for the ability to activate Src. We found that deletion of the internal unique domain of Cas (CasΔUR), like the full-length protein, had little effect on the ability of this protein to activate transcription (Fig. 3A and B, lanes 5). The lack of an effect on transcriptional activation by Cas and CasΔUR correlated with little or no effect on Y416 phosphorylation and therefore Src activation (Fig. 2B, lanes 4 and 5), despite the fact that these proteins associated with and were phosphorylated by Src (Fig. 2A, lanes 4 and 5).

Introduction of an additional deletion that removed the C terminus of Cas (CasΔURΔC) resulted in a protein that induced transcription of both SRE and AP-1 promoters to levels similar to that of full-length Sin (10-fold [Fig. 3A and B, lanes 6]). This suggests that, as for Sin, the conserved C terminus of Cas is involved in the negative regulation of the molecule. However, although this protein grossly resembled SinΔC and activated Src to the same extent as SinΔC (Fig. 2B, lanes 3 and 6), it was less potent than SinΔC in inducing transcription. To exclude the possibility that this effect was due to the lower affinity of the Cas proline-rich motif for the Src SH3, we exchanged the proline motif of Cas for that of Sin (CasΔURΔCP* [Fig. 1A]). This substitution had no greater effect on Src-dependent transcriptional activation than unmodified CasΔURΔC, suggesting that the Sin and Cas proline-rich motifs exhibit similar affinities for the Src SH3 domain (Fig. 3A and B, lanes 7). As with phosphorylation of the different Sin and Cas proteins, we found that the effect of these proteins on transcriptional activation was dependent on c-Src, since no induction of the SRE- and AP-1–luciferase reporters was ob-

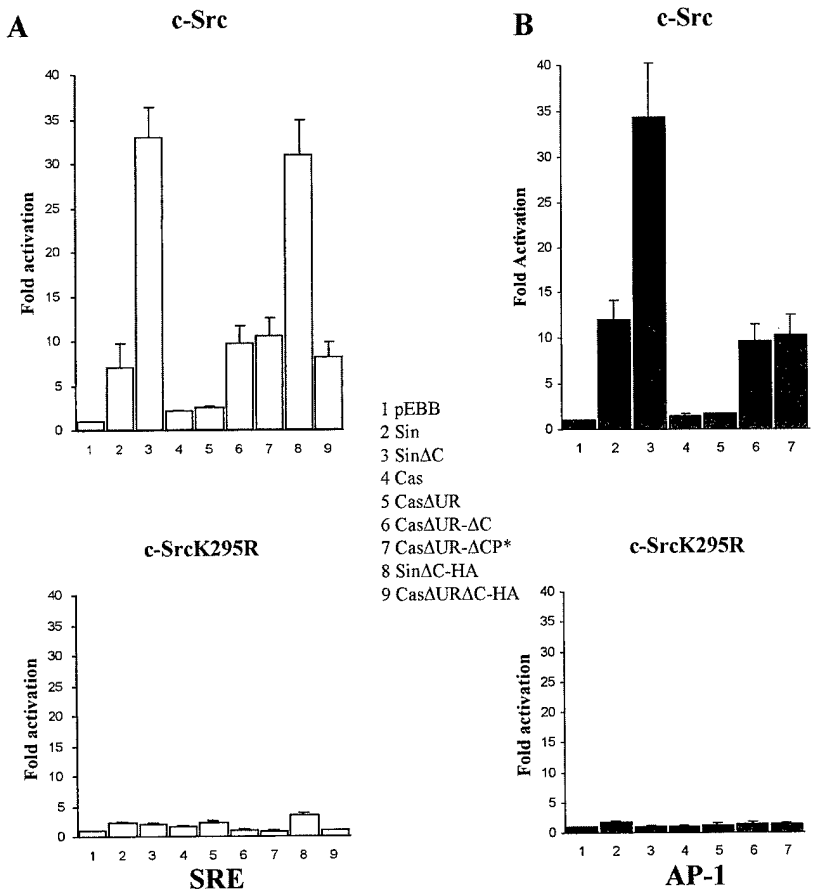


FIG. 3. The Sin and Cas proteins mediate differential activation of Src signaling. 293 cells were cotransfected as in Fig. 2 with a Sin or Cas expression construct (as shown, lanes 2 to 9) and wild-type c-Src (A and B, top) or the Src kinase mutant (A and B, bottom); 1 μg of luciferase reporter construct containing the SRE (left) or AP-1 (right) promoter was included in the transfection mix, along with β-galactosidase expression plasmid (1 μg). Luciferase activity was measured as described in Materials and Methods. The results represent the average of at least five experiments and are expressed as fold activation relative to the values obtained with the vector backbone (pEBB) that was used to express Sin and Cas proteins and was given a value of 1. The results shown represent the mean ± standard deviation.

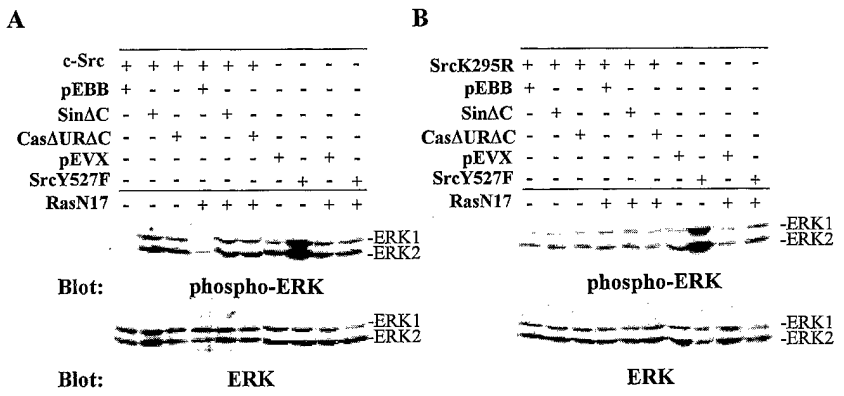
served in the presence of the SrcK295R kinase mutant (Fig. 3A and B, bottom).

Our data showing that deletion of the C termini of Sin and Cas leads to increased Src activation (Fig. 2A and B) suggest that Sin and Cas may themselves be regulated through intra- or intermolecular interactions. Consistent with this observation, addition of GST or an HA tag to either the N or C terminus of Sin gives rise to a full-length Sin protein that activates Src to levels similar to those observed with the truncated Sin protein (reference 1 and unpublished observations). Thus, to examine functional differences between full-length and truncated Sin and Cas proteins, structural modifications as a result of epitope tagging were avoided. However, since the truncated Sin and Cas proteins are already deregulated, we generated HA-tagged SinΔC and CasΔURΔC and tested their ability to bind to and activate Src signaling. We found that although the two molecules were expressed at similar levels (Fig. 2C, upper panel, lanes 8 and 9) and associated with Src to similar extents (Fig. 2A, upper panel, lanes 8 and 9), CasΔURΔC was still less potent than SinΔC in activating SRE-dependent transcription (Fig. 3A, lanes 8 and 9). Increases in the concentration of transfected plasmid DNA expressing different Cas proteins had no additional effect on their ability to induce Src signaling (data not shown). Thus, these results show that although all of the proteins we used associate with and are phosphorylated by Src, they differ quantitatively in the ability to activate Src and mediate Src signaling.

Sin and Cas-induced Src signaling is independent or downstream of Ras. Given the quantitative differences between Sin and Cas, we next examined whether these proteins utilize the same or different pathways to propagate Src signaling. It has been previously shown that the small GTP-binding protein Ras mediates signaling by constitutive active and oncogenic Src alleles (26, 51, 67). Thus, we further evaluated the pathway(s) that is activated as a result of Sin and Cas binding to Src and examined whether Ras also plays a role in Sin-Cas-induced Src signaling.

Tyrosine kinase-stimulated cell growth and proliferation in response to growth factor receptor stimulation induces the activity of the Ras proto-oncogene and the extracellular signal-regulated kinases ERK1 and -2 (23, 49, 71, 91). To assess the role of Ras and ERK in ligand-mediated Src signaling, we used a well-characterized dominant negative inhibitor of Ras (RasN17) (30) and examined its effect on Sin-Cas-induced and Src-dependent ERK1,2 activation. For these experiments we used proteins that were the most active in inducing transcriptional activation, namely, SinΔC and CasΔURΔC (Fig. 1; Fig. 3A and B, top).

Using an antibody that specifically recognizes the phosphorylated, active forms of ERK1,2, we found that both SinΔC and



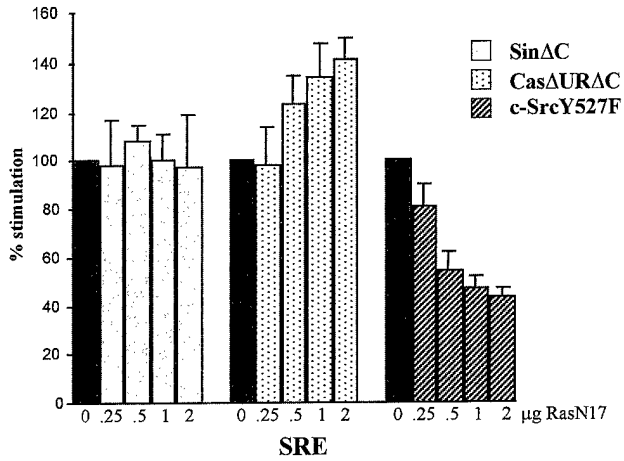


FIG. 4. Induction of ERK1,2 phosphorylation and SRE-dependent transcriptional activation by Cas and Sin are Ras independent. (A) 293 cells were transfected with c-Src and pEBB vector or SinΔC or CasΔURΔC as shown. Also cells were transfected with SrcY527F alone, or empty vector used to express this Src mutant (pEVX), in the presence of the Ras inhibitor (2 μg) or the Zipneo empty vector (2 μg) as shown. Total cell extracts were normalized for protein content, resolved by SDS-PAGE, and blotted with anti-phospho-ERK antibody (E-4) (top panel). The lower panel represents a Western blot probed with anti-ERK antibody that recognizes both phosphorylated and unphosphorylated forms of ERK. (B) 293 cells were transfected as above but in the presence of the Src kinase mutant or SrcY527F as a positive control and in the presence of RasN17 inhibitor or empty vector. Western blots of cell extracts were processed as in panel A. (C) Cells were transfected with c-Src and SinΔC or CasΔURΔC or with SrcY527F alone, as shown, with increasing concentrations of the RasN17 inhibitor in the presence of the SRE-luciferase reporter. Percent stimulation is relative to the activation of luciferase in cells transfected with c-Src and SinΔC or CasΔURΔC, or SrcY527F in the presence of the Zipneo empty vector (2 μg) used to express the RasN17 inhibitor, each of which was given a value of 100 (dark gray bars). Actual fold activation in the absence of the RasN17 inhibitor (dark gray bars) was 32 ± 3.2 for SinΔC, 14 ± 2.4 for CasΔURΔC, and 79 ± 16 for SrcY527F. The percent stimulation obtained with c-Src and SinΔC or with CasΔURΔC or SrcY527F in the presence of the dominant negative inhibitors is the mean ± standard deviation. The data represent the average of at least five experiments.

CasΔURΔC induced ERK phosphorylation when coexpressed with c-Src (Fig. 4A, top panel). However, the induction of ERK phosphorylation by SinΔC and CasΔURΔC was not sensitive to the RasN17 dominant negative inhibitor, suggesting that the activation of ERK1,2 was downstream or independent of Ras (Fig. 4A, top panel). This result is in contrast to published observations showing Ras to be the main upstream positive regulator of ERK (23, 49, 71, 91). In addition, these results differ from published data that show Ras to act downstream of oncogenic *src* alleles (26, 51, 67). Consistent with the published observations, in our system we found that activation of ERK1,2 by the constitutively active Src mutant (SrcY527F) was blocked by the RasN17 inhibitor (Fig. 4A, top panel). The effects of Sin and Cas on increased ERK1,2 phosphorylation were specific given that probing the blots with an ERK antiserum which recognizes both the phosphorylated and nonphosphorylated forms of the kinases revealed similar levels of the proteins (Fig. 4A, bottom panel). The effect of SinΔC and CasΔURΔC on ERK1,2 activation was Src dependent, since coexpression of the SrcK295R kinase mutant with SinΔC and CasΔURΔC did not induce ERK phosphorylation (Fig. 4B, top panel). In the same experiment, expression of the constitutively active SrcY527F mutant resulted in ERK1,2 activation; ERK1,2 phosphorylation was again blocked by the RasN17 inhibitor. Equal levels of total ERK were present in all lanes (Fig. 4B, bottom panel). Our data therefore suggest that the Sin and Cas proteins activate ERK1,2 independently or downstream of Ras and that this effect is dependent on Src kinase activity.

In addition to ERK activation, we also tested the effect of the RasN17 inhibitor on SRE-dependent transcriptional activation. Consistent with the ERK activation described above, SinΔC and CasΔURΔC-mediated, Src-dependent SRE activation was not blocked by the Ras inhibitor (Fig. 4C), whereas activation of the SRE-luciferase reporter by the constitutively active SrcY527F mutant was inhibited in a concentration-dependent manner (~60% inhibition [Fig. 4C]). Thus, these data show that activation of ERK and SRE-mediated transcription as a result of SinΔC and CasΔURΔC binding to c-Src occur through a mechanism that is Ras independent. In addition, these results suggest that signaling through Sin-Cas-activated Src and signaling through constitutively active Src proteins are mediated by different mechanisms.

Sin- and Cas-induced Src signaling is mediated by Crk. In vitro studies have previously shown that a phosphorylated tyrosine within a YDVP consensus sequence (Y motif) exhibits preferential binding to the c-Crk SH2 domain (78). c-Crk is a Grb2-like small adapter molecule with no known enzymatic activity, consisting mainly of modular regions such as SH2 and SH3 domains (31). Three of the Sin Y motifs and the majority of the Cas Y motifs are of the YDVP consensus, suggesting a c-Crk SH2 binding specificity (Fig. 1A). Consistent with the in vitro data, Sin and Cas interact with endogenous c-Crk in a c-Src- and phosphotyrosine-dependent manner (1, 72).

To determine whether the association of phosphorylated SinΔC and CasΔURΔC proteins with endogenous c-Crk was functionally significant, we tested whether an SH3 domain mutant of Crk could inhibit Sin-Cas-induced transcriptional activation. This c-Crk mutant has been previously described and was used to successfully block activation of ERK1 by oncogenic v-Abl (81). The W170K point mutation within the SH3 domain of Crk acts by interfering with binding to downstream proline-rich containing effector molecules such as the guanine nucleotide exchange factor (GEF) C3G (82). As shown in Fig. 5A, the Crk mutant effectively inhibited SRE-dependent transcriptional activation by SinΔC and CasΔURΔC in a concentration-dependent manner, suggesting that c-Crk is required for Src signaling mediated by these proteins. The effects of the Crk inhibitor on Sin-Cas-induced Src signaling were specific, particularly when lower concentrations of inhibitor DNA were used. A small inhibition of v-Raf-induced SRE-luciferase activation was observed at the highest concentration of the inhibitor (2 μg), suggesting a nonspecific effect possibly due to toxicity (Fig. 5A). Similar results were obtained using the AP-1 promoter (data not shown).

The experiments described above suggest that Src signaling is dependent on the substrates recruited by the conserved Y motifs of Sin and Cas. To further examine whether Crk recruitment by the YDVP motifs mediates the signaling effects of Sin, we introduced point mutations in the three YDVP motifs of SinΔC, at positions Y148, Y188, and Y253. We concentrated on Sin because it contains fewer YDVP motifs than Cas. We found that double mutants containing different combinations of mutagenized tyrosine residues (as shown) or a single mutation on Y253 significantly blocked the ability of SinΔC to activate SRE-dependent transcription (Fig. 5B). In addition, a mutant containing substitutions on all three tyrosines (Y148, Y188, and Y253) inhibited about 90% of Src-induced transcriptional activation (Fig. 5B). Similar results were also obtained with the AP-1 reporter construct (data not shown).

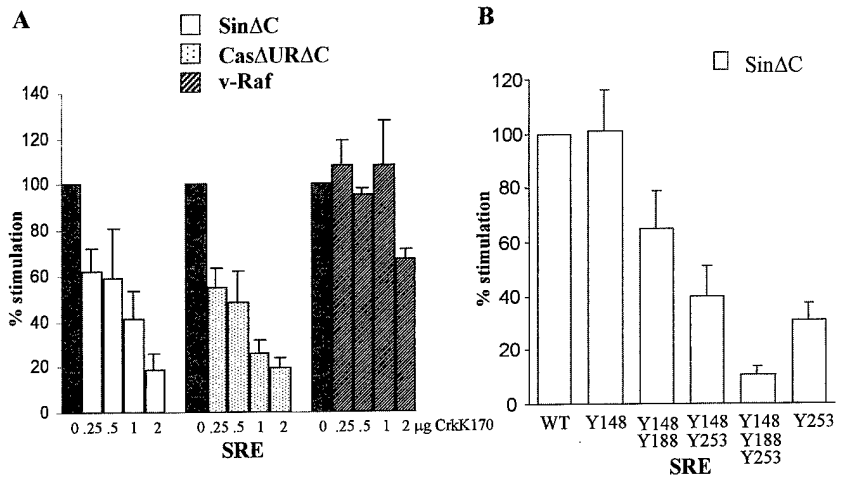
The ability of the triple mutant to block transcription correlated with reduced phosphorylation of this protein by Src (Fig. 5C, middle panel, lane 6). This was despite the fact that the mutant was expressed to levels similar to those observed with wild-type SinΔC (Fig. 5C, upper panel, lane 6) and associated with Src, albeit to a lesser extent than SinΔC (Fig. 5C, lower panel, lane 6). These results show that mutations within virtually identical motifs of SinΔC can behave differently in terms of mediating the effects of Src. These differences may be due to amino acids N terminal to the tyrosine that are involved in determining kinase specificity. Indeed, in vitro studies have shown that the presence of acidic amino acids (D or E) at positions −4 and −3 N terminal to an unphosphorylated tyrosine residue correlates with Src-kinase specificity (77) (Fig. 5C, bottom). Taken together, these data suggest that Crk recruitment by the YDVP motifs of Sin is important for Sin-mediated Src signaling.

The Rap1 GTPase is involved in Cas- and Sin-induced Src signaling. It has been recently shown that the SH3 domain of c-Crk interacts with proline-rich sequences found on C3G (34, 46, 82), a protein that promotes nucleotide exchange on the small GTP-binding protein Rap1 (38). In our system, we also found a Src-dependent association of endogenous Crk and C3G with phosphorylated SinΔC and CasΔURΔC (Fig. 6A). These interactions were dependent on the kinase activity of Src since the association of Sin and Cas with Crk and C3G was abolished in the presence of the Src kinase mutant (data not shown).

To address whether Rap1 was a downstream effector for Crk in our system, we used a dominant negative mutant of Rap1 that is analogous to the RasN17 inhibitor and blocks activation of the endogenous Rap1 protein (86). We found that, like the Crk inhibitor, Rap1N17 blocked SinΔC- and CasΔURΔC-induced transcriptional activation through the SRE-luciferase reporter, in a concentration-dependent manner (Fig. 6B). In the same experiments, SRE activation by v-Raf was largely unaffected, particularly at lower concentrations of the inhibitor, suggesting that the effect of the Rap1 inhibitor is specific (Fig. 6B). Therefore, these results demonstrate that Rap1 acts downstream of phosphorylated SinΔC and CasΔURΔC to mediate Src signaling.

To further examine the involvement of Rap1 in Sin- and Cas-mediated Src signaling, we tested whether Src and SinΔC or CasΔURΔC coexpression resulted in increased levels of active, GTP-bound Rap1. For this purpose we used a recently developed technique that involves the use of the GST–Ral-GDS-RBD fusion protein in affinity binding assays. This molecule contains a fragment of the Rap1 effector RalGDS, which specifically binds to the effector-binding domain of activated, GTP-bound Rap1 (32, 90). We found that coexpression of SinΔC or CasΔURΔC with c-Src resulted in increased levels of active, GTP-bound Rap1 (Fig. 6C, upper panel). In the same experiment, we tested whether Ras was activated as a result of Sin-Cas-mediated activation of Src. To this end, we used GST fused to c-Raf-RBD, which contains a fragment of the Ras-binding domain of c-Raf, which is one of the downstream effector molecules for Ras (21). c-Raf-RBD specifically recognizes active GTP-bound Ras but not Rap1. We found that, in contrast to Rap1, coexpression of c-Src with SinΔC or CasΔURΔC did not induce Ras activation (Fig. 6C, bottom panel). This data are consistent with results for the dominant negative Ras and Rap inhibitors and further support the model that Sin- and Cas-induced activation of Src signaling is mediated exclusively by Rap1. These data further indicate that different signaling mechanisms are utilized by ligand-activated versus constitutively active Src.

Rap1 was originally discovered as a transformation suppressor of Ki-Ras (43), and it has been shown to antagonize Ras function in different cell systems (9, 10). However, in other cell types Rap1 has also been shown to have positive effects, i.e., induce proliferation and growth in Swiss 3T3 cells (29, 96),



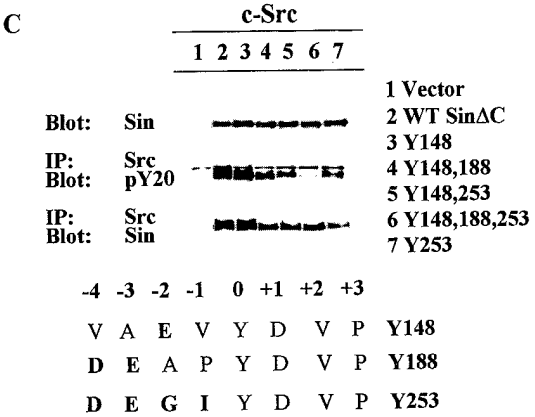


FIG. 5. The adapter Crk mediates Sin- and Cas-induced Src signaling. (A) 293 cells were cotransfected with c-Src (2 μg) and SinΔC or CasΔURΔC (1 μg of each) or with a v-Raf expression plasmid (2 μg) in the presence of empty pEBB vector or increasing concentrations of the CrkK170 inhibitor. Percent stimulation is relative to the activation of luciferase in cells transfected with c-Src and SinΔC or CasΔURΔC, or v-Raf in the presence of the pEBB empty vector (2 μg) used to express the CrkK170 inhibitor, each of which was given a value of 100 (dark gray bars). Actual fold activation in the absence of the CrkK170 inhibitor (dark gray bars) was 39 ± 11 for SinΔC, 12 ± 2.8 for CasΔURΔC, and 126 ± 15 for v-Raf. The percent stimulation obtained with c-Src and SinΔC or with CasΔURΔC or v-Raf in the presence of the dominant negative inhibitors is the mean ± standard deviation. The data represent the average of at least seven experiments. (B) 293 cells were cotransfected with c-Src and wild-type (WT) or mutant SinΔC (1 μg, as shown) in the presence of the SRE-luciferase reporter construct. Data from several experiments ($n = 5$) were averaged, and values for samples that expressed the mutant Sin proteins were normalized to the induction of luciferase activity observed with wild-type SinΔC, which was given a value of 100 (actual increase with wild-type SinΔC was 42 ± 4.7-fold). The percent stimulation is the mean ± standard deviation. (C) Lysates of 293 cells expressing c-Src and wild-type or tyrosine mutant SinΔC were immunoprecipitated (IP) with anti-Src antibodies (middle and bottom panels). Immunoprecipitates were blotted and probed with antiphosphotyrosine antibody pY20 (middle) or anti-Sin antibody (bottom). Total cell extracts of the same samples were resolved by SDS-PAGE, and the membranes were blotted with anti-Sin antibody (top panel). Consensus sequences containing Y148, Y188, and Y253 are shown at the bottom of panel B. Amino acids important for kinase specificity are shown in bold.

activate intermediates of the mitogen-activated protein (MAP) kinase cascade in PC12 cells (86, 95), and mediate bombesin-induced phosphorylation of ERK (66). These observations are consistent with our results showing that Rap1 and ERK are activated in response to ligand-induced activation of Src. To show that ERK can be directly activated in our system, we expressed a constitutively active form of Rap1, Rap1V12, or wild-type Rap1 in 293 cells and assayed for increases in ERK1,2 phosphorylation. We found that expression of either RapV12 or wild-type Rap1 leads to increased ERK phosphorylation (Fig. 6D, upper panel), while equal levels of total ERK were observed in all lanes (Fig. 6D, lower panel). Thus, these data suggest that overexpressed Rap1 can indeed activate ERK in 293 cells.

We next examined the effect of the different inhibitors on ligand-induced JNK/stress-activated protein kinase (SAPK)-mediated, AP-1-dependent gene expression. We found that, as with the SRE promoter, AP-1-dependent transcriptional activation by SinΔC and CasΔURΔC was Ras independent and was instead mediated by Crk and Rap1 (data not shown). However, we were unable to detect direct activation of the JNK/SAPK protein using either an antibody against the phosphorylated form of this kinase or in vitro phosphorylation of GST–c-Jun (data not shown). In addition, dominant negative inhibitors of intermediates of the JNK pathway such as JNKK1 and JNK had no effect on AP-1-mediated gene expression. However, a dominant inhibitor of MEK-1 blocked both SRE- and AP-1-mediated transcriptional activation by all three ligands (data not shown). Thus, it appears that activation of gene expression through the

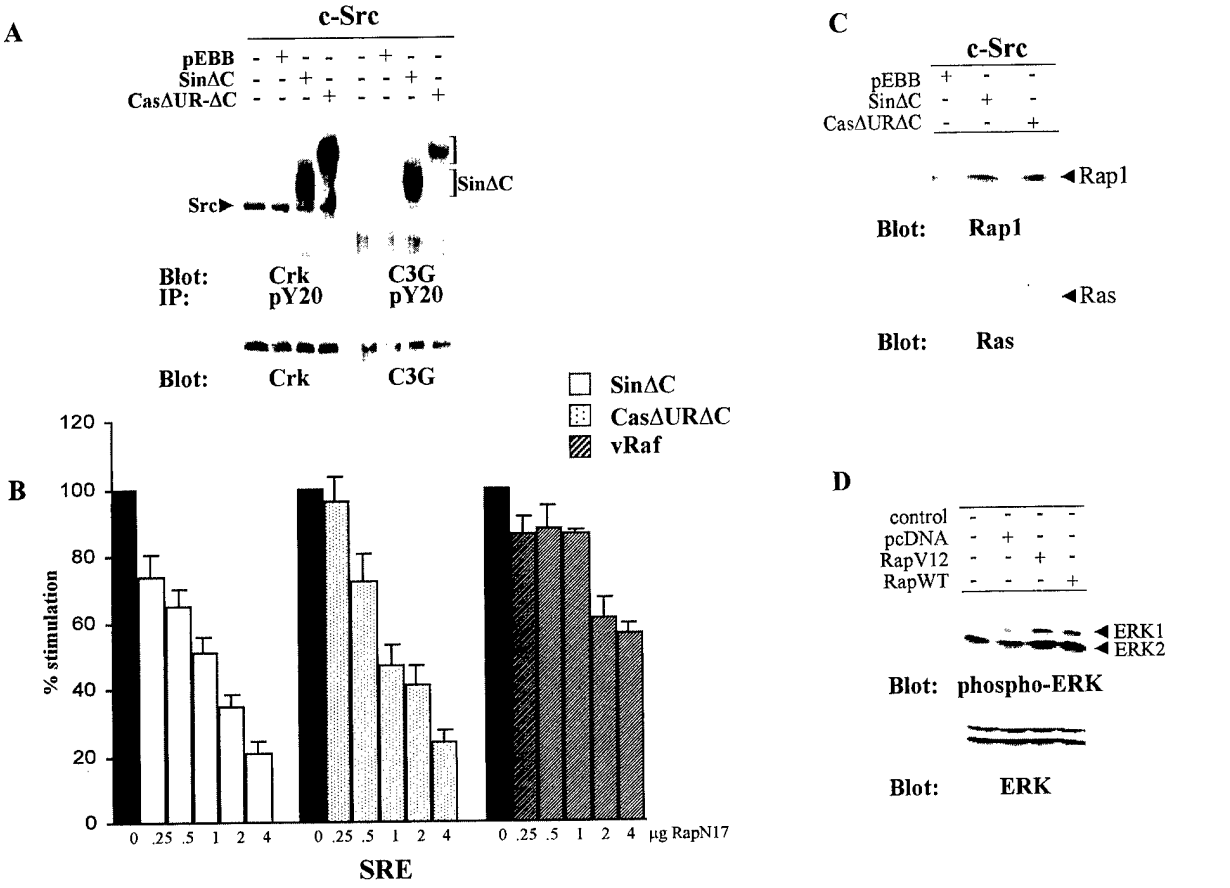


FIG. 6. Sin- and Cas-induced Src signaling is mediated by the Rap1 GTPase. (A) 293 cell lysates expressing c-Src and SinΔC or CasΔURΔC were immunoprecipitated with Crk-specific (left) or C3G-specific (right) antibodies. Immunoprecipitates (IP) were blotted and probed with antibody pY20. Membranes were then stripped and blotted with anti-Crk (bottom left) or anti-C3G (bottom right) antibodies. (B) 293 cells were transfected as in Fig. 5A along with increasing concentrations of the RapN17 inhibitor as shown. Percent stimulation is relative to the activation of luciferase in cells transfected with c-Src and SinΔC or CasΔURΔC, or v-Raf in the presence of the pcDNA empty vector (4 μg) used to express the Rap1N17 inhibitor, each of which was given a value of 100 (dark gray bars). Actual fold activation in the absence of the RapN17 inhibitor (black bars) was 26 ± 6.5 for SinΔC, 10 ± 3.3 for CasΔURΔC, and 86 ± 5.2 for v-Raf. The percent stimulation obtained with c-Src and SinΔC or with CasΔURΔC or v-Raf in the presence of the dominant negative inhibitors is the mean ± standard deviation. The data represent the average of at least six experiments. (C) 293 cell extracts expressing c-Src and vector backbone, SinΔC, or CasΔURΔC were incubated with GST–RalGDS-RBD or GST–Raf-RBD; the protein complexes were harvested, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blotted with Rap1-specific (top) or Ras-specific (bottom) antibodies. Protein bands were visualized using ECL. (D) Total extracts from untransfected or cells expressing pcDNA vector, constitutively active RapV12, or wild-type Rap (RapWT) were blotted with anti-phospho-ERK (top) or anti-ERK (bottom). Protein bands were visualized using ECL.

AP-1 site is not through independent activation of the JNK pathway but rather the result of ERK-mediated gene expression.

Signaling through the constitutively active SrcY527F mutant is mediated by both Ras and Rap1. The lack of an inhibitory effect with the Ras inhibitor on Sin and Cas induced activation of c-Src is surprising in that SRE-mediated transcription in response to tyrosine kinase activation is predominantly the result of Ras-mediated activation of the ERK1,2 MAP kinases (23, 49, 71, 91). It has also been shown that transcriptional activation induced by constitutively active and oncogenic forms of Src is Ras dependent (45, 63, 67). Consistent with these observations, we found that the Ras inhibitor blocked SrcY527F-induced SRE activation (Fig. 4C). We next examined whether the SrcY527F constitutively active mutant could activate the Crk-Rap1 signaling pathway, using dominant negative inhibitors and assaying for Rap activation. We found that both the CrkK170 and RapN17 mutants inhibited SrcY527F-induced transcriptional activation of the SRE promoter in a concentration-dependent manner (Fig. 7A), suggesting that both the Ras and Rap1 signaling cascades are utilized by oncogenic Src. Consistent with this result, cotransfection of SrcY527F with both RasN17 and RapN17 resulted in maximal inhibition of transcription even at the lowest concentrations of Rap and Ras inhibitor DNA (0.25 μg of each [Fig. 7A]). In addition, in contrast to Sin and Cas, which preferentially activated Rap1 but not Ras, we found that expression of the SrcY527F mutant resulted in increased GTP-bound levels of both Ras and Rap1 (Fig. 7B). Furthermore, as with the RasN17 inhibitor (Fig. 4A), we found that SrcY527F-mediated activation of ERK1,2 phosphorylation was also sensitive to the RapN17 inhibitor, whereas v-Raf-induced ERK phosphorylation was unaffected under the same conditions (Fig. 7C). Thus, these data suggest that the oncogenic SrcY527F mutant utilizes at least two separate cascades to ERK phosphorylation and SRE promoter activation, whereas signaling through ligand-activated Src is more directed and is primarily mediated by Rap1.

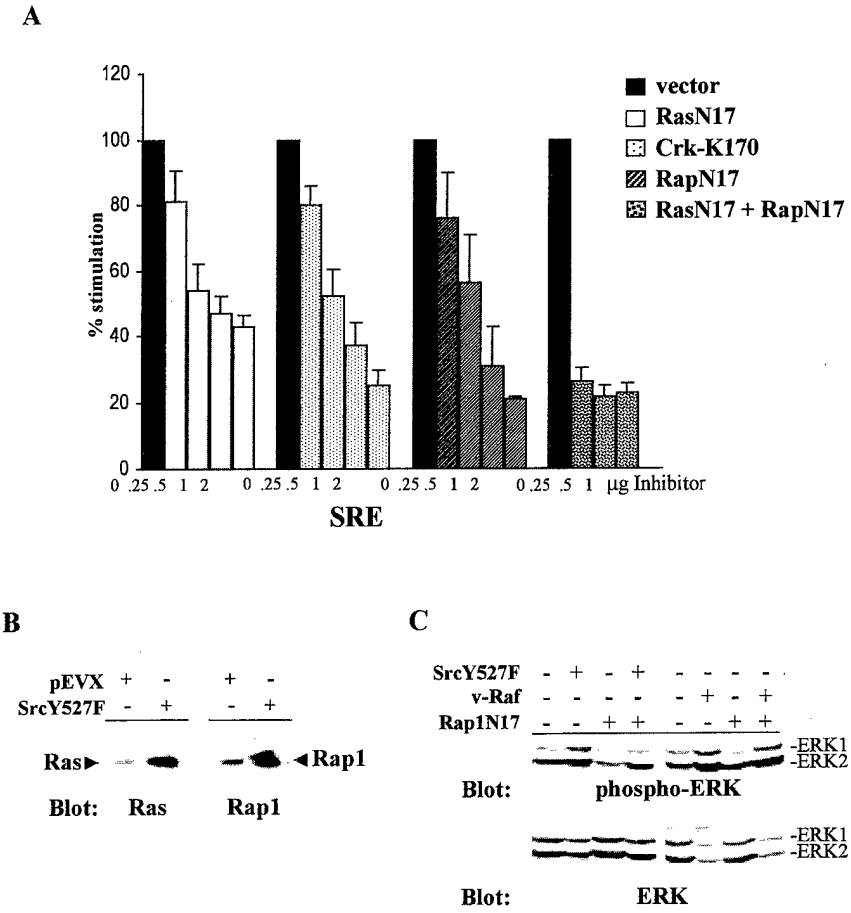


FIG. 7. Signaling through the constitutively active SrcY527F mutant is mediated by both Ras and Rap. (A) 293 cells were transfected with SrcY527F in the presence of the Ras, Crk, and Rap inhibitors and in the presence of the SRE-luciferase reporter. Percent stimulation represents the average data from four experiments and was determined from samples expressing SrcY527F in the presence of empty vectors (pZipneo, pEBB, and pcDNA) that were used to express the inhibitors (dark gray bars). Actual activation was 79 ± 16-fold in the presence of Zipneo, 130 ± 28-fold for pEBB, and 40 ± 6.7-fold for pCDNA. The difference in the SrcY527F fold activation observed in the presence of the different vectors is likely due to promoter competition of the transfected plasmids. Luciferase activation in the presence of the different inhibitors is expressed as a percentage of the dark gray bar controls ± standard deviation. (B) Cell extracts expressing the pEVX vector or SrcY527F were incubated with GST–RalGD-RBD or GST–Raf-RBD; protein complexes were separated, transferred to membranes, and blotted with Rap- or Ras-specific antibodies, respectively. Protein bands were visualized using ECL. (C) 293 cell extracts expressing SrcY527F or v-Raf were assayed for ERK1,2 activation in the presence of the RapN17 inhibitor. SrcY527F or v-Raf (2 µg of each) and RapN17 (4 µg) expression plasmids were used. Cell lysates were analyzed as described for Fig. 4, using phospho-ERK- and ERK-specific antibodies.

DISCUSSION

Cas and Sin mediate Src-dependent transcriptional activation through the ERK MAP kinase. The Src-SH3 domain is important for c-Src regulation and signaling, and this domain is thought to participate in signaling specificity by determining the substrate selectivity of the enzyme (28). In this study we used the natural c-Src ligands Sin and Cas to activate Src and examine the mechanisms that mediate signaling under these conditions. In the experiments described above, we found that phosphorylated Sin and Cas act downstream of Src to activate transcription through an SRE-containing promoter. SRE-dependent transcriptional activation has been shown to depend on activation of Ras and the ERK1,2 MAP kinases. In our system, activation of transcription through the SRE was Ras independent and was instead mediated by the related protein Rap1. Our results for the first time implicate Rap1 in Src kinase signaling and show that this G protein acts downstream of phosphorylated Sin and Cas to activate ERK and SRE-dependent transcription.

Cas has been the focus of many studies, which show this protein to mediate a variety of cellular processes. Recent evidence suggests that extracellular signals, such as integrin engagement, lead to the formation of an active Cas-Crk signaling complex, which regulates cell migration and survival (17, 41), cell cycle progression (60), and transcriptional activation (24, 35). These responses are mediated by the ERK and JNK MAP kinase cascades, which are activated by distinct, upstream-acting signaling complexes. Thus, activation of the ERK1,2 kinases in response to extracellular stimuli is mediated by the Crb2-Sos or the Shc–Grb-2–Sos signaling modules, whereas activation of the JNK MAP kinase is mediated by the formation of an active Cas-Crk complex (17, 60, 97). In the integrin receptor system, Cas-Crk coupling is facilitated by upstream-acting Src and FAK, and this coupling results in the activation of the small GTP-binding protein Rac. Activation of Rac, in turn, regulates JNK activation and cell invasion and adhesion (17). In our system, coupling of Cas and Sin with Crk as a result of Src-mediated phosphorylation of Cas and Sin induced ERK but not JNK activation. In addition, a dominant negative mutant of Rac was not able to block Cas-Sin-induced SRE- or

AP-1-dependent transcriptional activation (unpublished observations).

The lack of an effect of Src-mediated phosphorylation of Sin-Cas and Crk coupling on JNK activation was unexpected given existing evidence suggesting that Src-induced Cas phosphorylation in response to integrin stimulation mediates integrin-dependent JNK activation (8, 60, 74, 87). However, to our knowledge there is no evidence that directly links Src-mediated Cas phosphorylation to JNK activation. On the other hand, consistent with our results showing that Cas and Sin are not involved in Src-dependent JNK activation is evidence suggesting that a dominant negative inhibitor of Cas does not inhibit v-Src-induced JNK activation (24). It is possible therefore, that other kinases act downstream of Src to phosphorylate Cas and activate JNK in response to integrin stimulation. Indeed, it has been shown that although Cas phosphorylation is abolished in $Src^{-/-}$ cells, expression of a truncated, kinase-deficient Src protein in the same cells restores Cas phosphorylation and promotes Cas and FAK association and FAK-mediated phosphorylation of Cas (74). These observations suggest that there are differences in the formation of signaling complexes in the case of activated Src (either oncogenic or ligand induced) and activated integrin receptors that may result in different signaling mechanisms through these proteins. Thus, additional elements may be recruited downstream of activated integrin receptors versus activated Src to induce JNK activation.

In contrast to the inability of the Cas and Sin proteins to activate JNK, we found that the ERK1,2 kinases were efficiently activated by these proteins in a Src-dependent manner (Fig. 4A and B). Although it has been suggested that Src-mediated phosphorylation of Cas may mediate ERK2 activation (74), the majority of existing evidence shows that Cas-Crk coupling in response to extracellular stimuli functions upstream of JNK (17, 60, 97). In addition, integrin- and cytokine-induced activation of ERK1,2 is mediated predominantly by the Grb2-Sos complex, and dominant negative Cas or Crk mutants do not block ERK activation (8). Furthermore, Src has been shown to mediate ERK activation in response to integrin stimulation either by promoting binding of Grb2-Sos to FAK or Pyk2 or by promoting formation of the Shc-Grb2-Sos complex (8). It has been previously suggested that Src may activate ERK through FAK-mediated phosphorylation of Cas in response to fibronectin, in the absence of Grb2 binding to FAK (74). Our experiments revealed that a dominant negative mutant of Ras had no effect on Sin- and Cas-mediated ERK phosphorylation and induction of transcription, whereas a dominant negative Crk mutant inhibited SRE-dependent transcriptional activation (Fig. 5), as well as ERK activation (data not shown). In the same experiments we also found that constitutively active Src can activate ERK through two distinct pathways: one that utilizes the Grb2-Sos-Ras cascade and another that involves the Crk-C3G-Rap1 signaling complex (Fig. 4 and 7C). Thus, it is possible that under different stimulating conditions these pathways either independently or in concert mediate ERK activation and need not be mutually exclusive. The experiments presented here show that the Cas-Sin-Crk-Rap1 pathway can indeed activate the ERK cascade and mediate signaling downstream of activated Src.

Src SH3-binding proteins differentially activate c-Src signaling. In the experiments described above, we found that although the different Sin and Cas proteins bound to and were phosphorylated by Src to similar levels, they elicited quantitatively different responses as measured by transcriptional activation. Despite these quantitative differences, both Sin and Cas mediated Src signaling through the activation of the Crk-Rap1-ERK1,2 signaling cascade.

The quantitative differences in transcriptional activation we observe with the Sin and Cas proteins do not appear to depend on differential binding affinities of these molecules for the Src SH3 domain. This is based on the observation that substitution of the Cas proline-rich motif with that of Sin did not increase the levels of transcriptional activation by CasΔURΔC (Fig. 3A and B, lanes 7). Alternatively, the quantitatively different effects of SinΔC and CasΔURΔC on transcription could be due to the tyrosine-containing motifs of these proteins. This hypothesis is supported by the observation that Sin-dependent transcriptional activation is dependent on specific YDVP motifs and that these motifs are not functionally equivalent (Fig. 5B). For example, Y188 and Y253 seem to have the strongest effect on the ability of Sin to mediate Src signaling (Fig. 5B). This correlates with the presence of amino acid positions −4 to −1 N terminal to Y188 and Y253 that contain the acidic amino acids D and E, which have been shown to be important for Src kinase specificity (Fig. 5C) (77). Moreover, the sequence upstream of Y253 (DEGI) is identical to the sequence described as optimal for Src kinase recognition by Songyang et al. (77) (Fig. 5C). In contrast, amino acids upstream of Y148 contain only one E residue (Fig. 5C), and a mutation on Y148 has no effect on transcriptional activation (Fig. 5B).

Although Cas contains more YDVP motifs than Sin, it was not as efficient as Sin in promoting Src signaling. This could be due to phosphorylation of a small subset of these motifs by Src, given that only three of the seven YDVP motifs of Cas contain D and E residues upstream of the tyrosine. Thus, the quantitative differences in transcriptional activation levels that we observe between Sin and Cas could be due to more efficient phosphorylation of some Sin motifs versus those of Cas. This, in turn, could result in more effective activation of downstream signaling intermediates. In support of this model, we observed that the mechanism of signaling (in terms of transcriptional activation) is the same for both Sin and Cas and is mediated by Rap1. However, it is also possible that Crk binding to the phosphorylated YDVP motifs of Sin may recruit other kinases that can then phosphorylate the unique Y motifs of Sin, thus contributing to the stronger effects of Sin on transcription. In future experiments, the use of the YDVP mutants in conjunction with additional mutations on other motifs will address these questions and may identify other individual elements that are important for Sin-mediated transcription.

c-Src signaling in response to ligand binding is mediated by Crk and Rap1, functioning upstream of ERK. The experiments presented above show that Cas and Sin can effectively activate the Crk-Rap1 signaling complex upstream of ERK and the SRE promoter and that this activation requires the binding of Crk to the phosphorylated YDVP motifs of Sin and Cas. Crk has been shown to bind to C3G through a Crk SH3 and C3G proline-rich motif interaction (82). Formation of the Crk-C3G complex in turn leads to Rap1 activation (34). In our experiments we found that both endogenous Crk and C3G associated with phosphorylated Sin and Cas proteins (Fig. 6A). These results suggest that phosphorylated Cas-Sin-induced activation of Rap1 is mediated by the Crk-C3G complex. It has been shown recently that Rap1 activation is mediated by different signaling pathways, involving second messengers such as calcium, diacylglycerol, and cyclic AMP (cAMP) (3, 32, 98). The formation of active, GTP-bound Rap1 is regulated by three different families of GEFs consisting of C3G, which regulates tyrosine kinase-induced Rap1 activation through Crk and Cbl (39, 69, 76), the guanine nucleotide-releasing proteins, which contain calcium and diacylglycerol motifs (25), and the Epac family of proteins, which are regulated by cAMP (22, 41). More recently, another protein family, the PDZ-GEFs, was

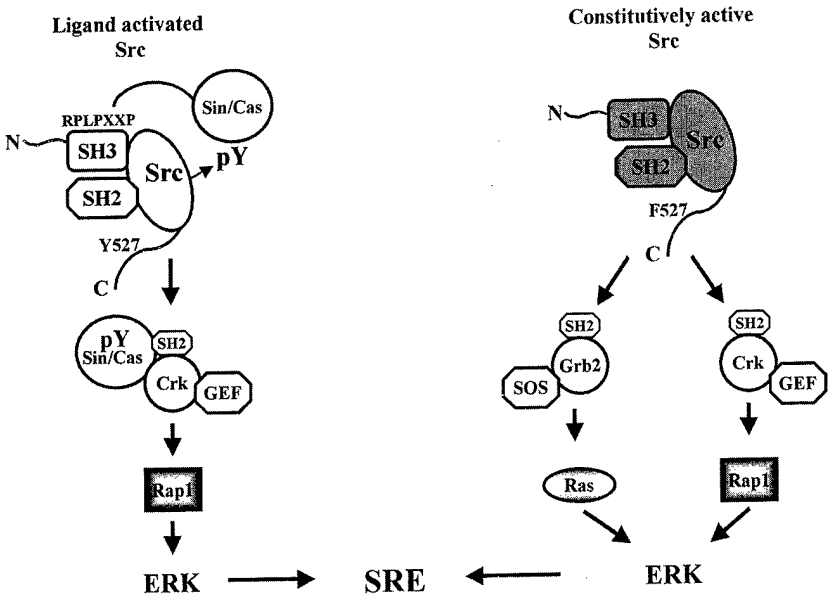


FIG. 8. Model for ligand-induced c-Src-dependent signal transduction leading to MAP kinase and transcriptional activation. Binding of the proline-rich motifs of Sin and Cas to the Src SH3 domain activates the c-Src tyrosine kinase activity, and the proteins become phosphorylated on tyrosine residues. Src-mediated phosphorylation of Sin and Cas results in the recruitment of the Crk-GEF signaling complex, which in turn activates Rap1 and ERK and induces SRE-mediated gene expression. On the other hand, mutations that disrupt the intramolecular interactions of Src, such as a point mutation on the C-terminal tyrosine 527, result in an open conformation of the enzyme, which then interacts nonspecifically with multiple cellular substrates. This, in turn, leads to the activation of at least two signaling cascades mediated by the Ras and Rap GTPases.

characterized, members of which exhibit nucleotide exchange activity specific for Rap1 and -2 (41). Thus, we cannot exclude the possibility that other, newly discovered Rap1-specific GEFs are involved in Rap1 activation in our system. The involvement of different GEFs in Rap1 activation will be explored in future experiments.

Rap1 was originally identified as the product of a cDNA (Krev-1) capable of suppressing transformation by Ki-Ras (43). More recently, both positive and negative functional outcomes have been described for activated Rap1 (see reference 6 for review). Thus, in addition to the transformation-suppressing effects of Rap1 (42, 43, 65), introduction of the active GTP-bound form of Rap1 into fibroblasts inhibits Ras-mediated activation of MAP kinase (19). Moreover, cAMP-dependent activation of Rap1 correlates with down-regulation of the MAPK-ERK pathway (27, 29, 70). On the other hand, other studies in different cell types indicate that Rap1 has additional functions besides being an antagonist of Ras signaling (9). Recently, Rap1 was shown to act synergistically with Ras in mediating nerve growth factor-induced differentiation of the phaeochromocytoma cell line PC12 (95) and to mediate ERK phosphorylation in response to cAMP (86). In addition, the activity of Rap1 has been shown to increase upon treatment of NIH 3T3 cells with the neuropeptide growth factor bombesin, and increased Rap1 activity correlates with increased phosphorylation of the MAP kinase ERK and increased proliferation (9, 66). Furthermore, Rap1 has been shown to induce DNA synthesis and oncogenic transformation in Swiss 3T3 cells (4, 96).

Rap1 is ~50% homologous to Ras, and the two proteins have effector domains that exhibit striking similarity (10). The high degree of homology of the Ras and Rap1 effector domains suggested that these proteins bind to the same effector molecules. Consistent with this model, Rap1 has been shown to interact with the Ras effector Raf1, although this interaction does not appear to lead to activation of this protein. Based on these data, it has been proposed that Rap1 may antagonize the function of Ras by competing for downstream effector molecules (19, 85). However, recent evidence suggests that growth factors that activate the Ras/Raf pathway also activate Rap1 and that this activation does not interfere with growth factor receptor signaling (98). In addition, increasing the levels of GTP-bound Rap1 by tetradecanoyl phorbol acetate does not inhibit ERK activation by platelet-derived growth factor and epidermal growth factor (98). In our system, activation of Rap1 correlates with ERK activation and increased gene expression. Furthermore, in the case of the SrcY527 mutant, Rap1 appears to act in concert with Ras to promote Src signaling. Taken together, these observations suggest that Rap1 function in cellular pathways may be more complex than previously thought and that its effect on cellular processes may be determined by cellular context.

Ligand-activated versus oncogenic Src signaling. In the experiments described above, we show that truncated Sin and Cas proteins mediate c-Src signaling through the Rap1 GTPase. These observations are important because (i) our results for the first time implicate a GTPase other than Ras in Src signaling, (ii) ERK activation can be mediated by small GTP proteins other than Ras, and (iii) the mode of Src activation (oncogenic versus ligand induced) may determine the signaling pathways activated by Src. The latter observation is particularly interesting given the fact that studies using constitutively active and oncogenic forms of Src have shown Src to act upstream of Ras (26, 51, 67). Consistent with this, our results show that signaling through the transforming allele SrcY527F is also Ras as well as Rap1 dependent (Fig. 7). In addition, a recent report by Hakak and Martin (35) showed that Cas mediates transcriptional activation of the Egr-1 SRE by v-Src and that this activation was through Grb2 and the Ras-MEK-ERK pathway. However, the increase in transcriptional activation was not dependent on the substrate region of Cas that contains the YXXP motifs, since deletion of this

region resulted in levels of luciferase activity equivalent to that induced by full-length Cas. Consistent with this observation, a Crk-II SH3 mutant had no effect on luciferase activity, suggesting that the effect of Cas on v-Src-dependent transcriptional activation is mediated by a Ras-dependent mechanism which is different from the one we are describing.

These observations support our model that the signaling mechanisms through activated forms of Src are more complex than the mechanisms that mediate ligand-induced Src signaling (Fig. 8). The differences that we observe in ligand-induced Src signaling versus signaling through constitutively active Src proteins could be due to signaling constraints imposed by the activating ligands through the recruitment of defined, downstream-acting sets of substrates. This stands in contrast to the broader spectrum of responses elicited by deregulated Src kinase activity and interactions between cellular substrates and transforming Src proteins. For example, v-Src as well as the Y527F Src proteins contain mutations that result in the disruption of intramolecular inhibitory interactions between the regulatory and kinase domains of Src (34, 45). This release of intramolecular inhibition abolishes the requirement for specific, high-affinity interactions of SrcY527F with putative substrates, resulting in a wider spectrum of cellular proteins interacting with and becoming phosphorylated by this mutant. As a result, multiple signaling pathways are activated. Indeed we found this to be the case in our system expressing the constitutive active SrcY527F in that this mutant activates at least two separate signaling cascades (Fig. 8).

In contrast to oncogenic alleles, activation of c-Src under physiologic conditions involves directed engagement of the conserved domains of c-Src by their ligands. For example, in response to growth factor binding, growth factor receptors activate Src by providing high-affinity ligands for the Src SH2 domain in the form of phosphotyrosines (83). This mechanism of activation excludes nonspecific binding of other proteins and ensures activation of specific signaling pathways for each receptor type. The use of Src SH3 ligands to activate c-Src more closely resembles the in vivo conditions, in that these ligands provide high-affinity binding sites for the Src SH3 domain. Once bound to Src, these ligands induce Src activation and subsequently serve as Src substrates and effector molecules. Given that each phosphorylated adapter molecule can attract a defined set of cytoplasmic intermediates, Src signaling through the recruitment of natural Src SH3 ligands may be more specific than signaling through constitutively active forms of Src. Whether coexpression of Src SH3 ligands and active Src alleles affects the signaling and transforming properties of oncogenic Src proteins remains to be determined.

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